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Diffuse Intrinsic Pontine Gliomas or DIPG, is a type of brain tumor that afflicts children and is the leading cause of death in pediatric brain tumor patients. One major obstacle to progress has been the lack of representative animal models that recapitulate the genetic alterations of the human disease in the appropriate cell-of-origin. Recent analysis of human DIPGs have unraveled the following key genetic alterations: K27M H3.3 or H3.1 mutations in 80% of tumors, p53 mutations in 75% of tumors, and focal amplification of components of the RTK/Ras/PI3K pathway in approximately 50% of tumors with 30% of tumors harboring amplification of PDGFRα. Using the RCAS/tv-a system, we have previously reported the development of a DIPG model by overexpression of PDGF-B in nestin progenitors of Ink4a-ARF deficient mice. Here we report the development of several improved DIPG models by overexpression of PDGF-B and Cre in nestin progenitors of conditional p53 deficient mice, conditional PTEN mice, and combined conditional p53 and PTEN mice. In addition we have also generated a new DIPG model by overexpression of PDGF-B and Cre in GFAP progenitors of conditional p53 mice. Lastly, to identify unique aspects of brainstem gliomagenesis we have compared the expression profile of the above described murine DIPG model to cortical gliomas induced by the same genetic alterations and unraveled that pax3 is a differentially expressed transcription factor that is upregulated in DIPGs and not in cortical gliomas.

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**Introduction**

Brain tumors are currently the leading cause of death for children with cancer. Diffuse Intrinsic Pontine Gliomas or DIPG, is a type of brain tumor that afflicts children and is the leading cause of death in pediatric brain tumor patients. One major obstacle to progress has been the lack of representative animal models that recapitulate the genetic alterations of the human disease in the appropriate cell-of-origin. Recent analysis of human DIPGs have unraveled the following key genetic alterations: K27M H3.3 or H3.1 mutations in 80% of tumors, p53 mutations in 75% of tumors, and focal amplification of components of the RTK/Ras/PI3K pathway in approximately 50% of tumors with 30% of tumors harboring amplification of PDGFRα (1-5). Using the RCAS/tv-a system, we have previously reported the development of a DIPG model by overexpression of PDGF-B in nestin progenitors of Ink4a-ARF deficient mice. Here we report the development of several improved DIPG models by overexpression of PDGF-B and Cre in nestin progenitors of conditional p53 deficient mice, conditional PTEN mice, and combined conditional p53 and PTEN mice. In addition we have also generated a new DIPG model by overexpression of PDGF-B and Cre in GFAP progenitors of conditional p53 mice. Lastly, to identify unique aspects of brainstem gliomagenesis we have compared the expression profile of the above described murine DIPG model to cortical gliomas induced by the same genetic alterations and unraveled that pax3 is a differentially expressed transcription factor that is upregulated in DIPGs and not in cortical gliomas. Goals for year 2 of this grant are to further improve the DIPG model by adding the K27M H3.3 mutation to the model, and to move closer towards unraveling the likely cell-of-origin for human DIPG by comparing the expression profile of DIPGs induced in nestin and GFAP expressing cells to the expression profile of human DIPGs. Lastly we will start using our improved DIPG model as a preclinical tool to help prioritize the translation of novel agents for clinical trials for children with DIPG.

Below is our current progress to date on the specific tasks as described in the Statement of Work. Overall, we are right on schedule with regards to our time-line.

**Task 1-Develop improved DIPG (Diffuse Intrinsic Pontine Glioma) mouse models that recapitulate human DIPG genetic alterations**

**Task 1a**- Infect the ventricular system of the posterior fossa of Nestin Tv-a; $p53^{fl/fl}$ (floxed/floxed) neonatal mice with RCAS-PDGF-B (**R**eplication-**C**ompetent, **A**vian leukemia virus [ALV] long terminal repeat [LTR], **S**plice acceptor- **P**latelet-**d**erived **g**rowth **f**actor- B) and RCAS-Cre (20 mice per group)

**This task has been completely successfully. We have generated these tumors and an example of one is illustrated in Figure 1. Out of 54 nestin tv-a; p53 floxed mice that were infected with PDGF and Cre, 46 developed murine DIPGs (85% success rate). Hydrocephalus mice were excluded from this calculation. With this particular experiment the hydrocephalus rate is 57% (due to the formation of leptomeningeal tumor). Figure 1 compares the tumor latency from Task 1a and Task 2c. Tumors formed in Task 1a are significantly more aggressive than tumors formed in Task 2c. Please note that we are including more than 20 mice per group in this survival analysis to demonstrate the difference between the two models. The additional tumor-bearing mice are not part of this award.**

**Figure 1**

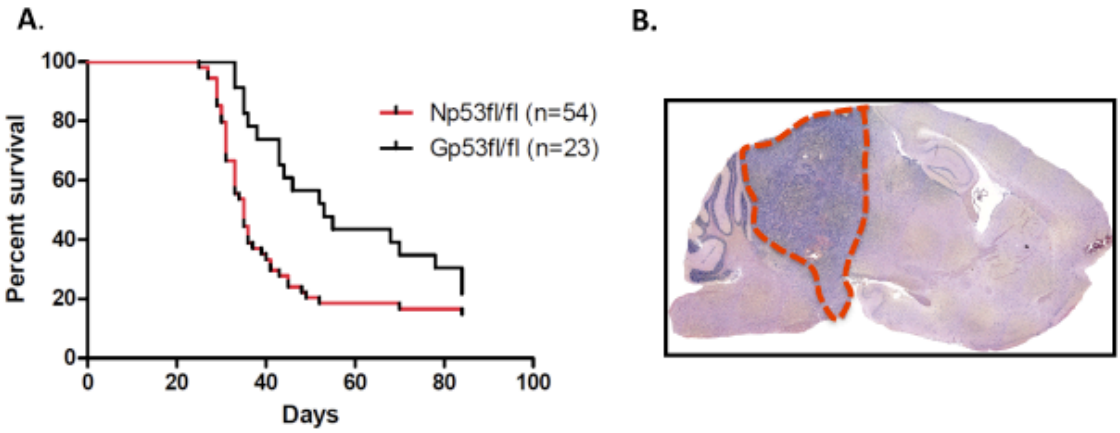



**Figure 1** A. Survival curves comparing tumor latency in the Nestin tv-a p53 floxed model and GFAP tv-a p53 floxed model. Log Rank test demonstrates that overexpression of PDGF-B with Cre in nestin progenitors results in more aggreessive DIPGs with shorter latency (p=0.019) B. Low magnification image of a DIPG induced in Nestin tv-a; p53 floxed mice. Red dotted line marks the tumor boundary.

**Task 1b**- Infect the ventricular system of the posterior fossa of Nestin Tv-a; $PTEN^{fl/fl}$ neonatal mice with RCAS-PDGF-B and RCAS-Cre (20 mice per group)

**This task is completed. We have generated 10 tumors by injecting 14 mice and an example of one is described in the figure below. Hydrocephalus mice were excluded from the**

**analysis. The hydrocephalus rate was 51% for this experiment due to the formation of leptomeningeal tumor.**

**Figure 2**

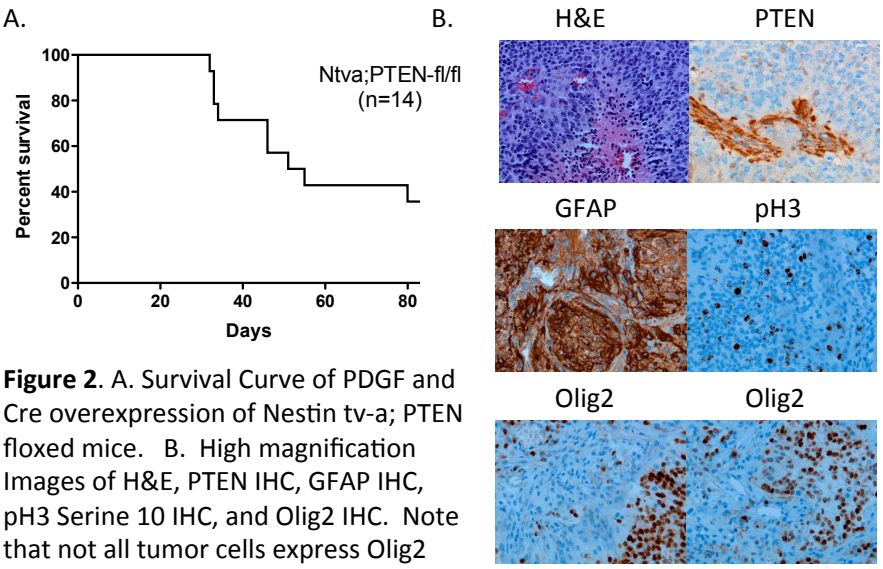



**Figure 2**. A. Survival Curve of PDGF and Cre overexpression of Nestin tv-a; PTEN floxed mice. B. High magnification Images of H&E, PTEN IHC, GFAP IHC, pH3 Serine 10 IHC, and Olig2 IHC. Note that not all tumor cells express Olig2

**Task 1c**- Infect the ventricular system of the posterior fossa of Nestin Tv-a; $p53^{fl/fl}$; PTEN $^{fl/fl}$ neonatal mice with RCAS-PDGF-B and RCAS-Cre (20 mice per group)
**This task has been partly completed. We have generated three such tumors and an example of one is described below (Figure 3). Hydrocephalus mice were excluded from the analysis. The hydrocephalus rate for this experiment was 63% (due to the formation of leptomeningeal tumor).**

**Figure 3**

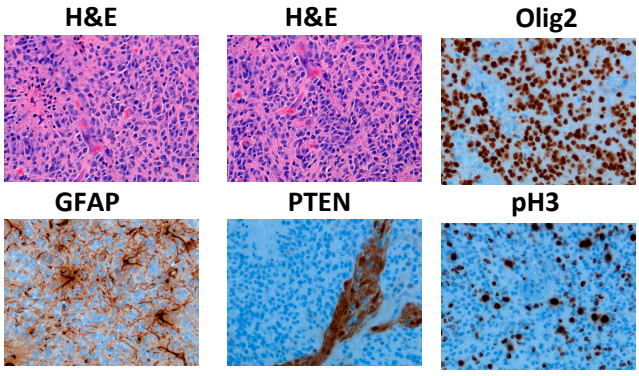



**Figure 3**. Representative H&Es and IHC for Olig2, pH3 (Serine 10), PTEN, GFAP. Note that PTEN staining in only present in the tumor vasculature

**Task 1d**- Analyze three tumors per genotype above by IHC (immunohistochemistry) for Ki-67, Cleaved caspase 3, GFAP (Glial Fibrillary Acidic Protein), nestin, Olig-2, SMA (Smooth Muscle Actin), and CD31.

**This task is partly completed. We had to change the proliferation marker for this task from Ki-67 to pH3 Serine 10 due to technical problems with the Ki-67 antibody (too much background staining). Representative IHC for task 1b and 1c are illustrated in Figure 2**

**and 3 respectively. Cleaved caspase 3, CD31, and SMA IHC of all tumor genotypes will be performed in year 2. For task 1a, representative IHC are in Figure 4 and Figure 6.**

Figure 4

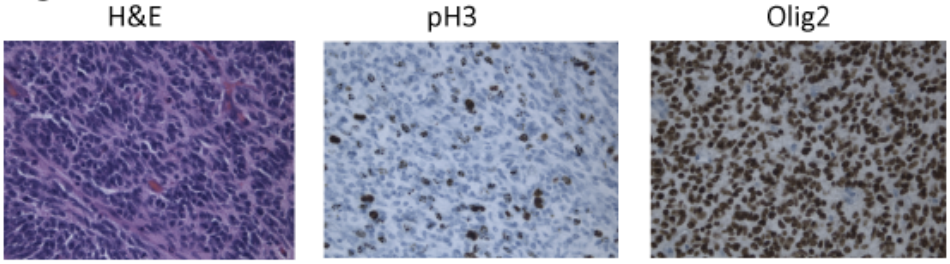



**Figure 4**. Representative H&E, pH3 Serine 10, and Olig2 of a PDGF; p53 deficient DIPG induced in nestin tv-a; p53 floxed mice.

**Task 1E**- Perform expression profiling of PDGF-B driven DIPG tumors of all the genotypes above (10 tumors per group)
**We have performed expression analysis of PDGF; p53 deficient tumors. Expression analysis of additional genotypes is ongoing and will be completed in year #2. A list of significant differentially expressed genes between the different tumor genotypes will be included in next year's annual progress report.**

**Task 1F**- Compare (in consultation with the DNA Microarray Facility at Duke) the murine expression profiles generated in Task 1E with to the expression profiling of human DIPGs that have PDGFRα (Platelet-derived growth factor receptor alpha) amplification (both from our analysis and published work)
**This task is ongoing and will be completed in year 2 of the award.**

**Task 1G**- Infect the ventricular system of the posterior fossa of Ntv-a (Nestin tv-a); Braf $^{LSLBrafV600E/wt}$ and Ntv-a;Braf $^{LSLBrafV600E/wt}$; ink4a-ARF-/- neonatal mice with RCAS-Cre and observe for the development of tumors (20 mice per group). LSL stands for Lox-Stop-Lox. BrafV600E refers to a mutation in the gene Braf where the amino acid E (Glutamic acid substitutes for the amino acid V (Valine).

**We are requesting to change this task to a new task 1G as sequencing of human DIPG did not reveal any Braf V600E mutations but instead identified a K27M mutation in H3.1 or H3.3 in the majority of DIPGs (1,4). New task is described below. The total mouse number will stay the same.**

**New Task 1G- Infect the ventricular system of the posterior fossa of Ntv-a (Nestin tv-a) with RCAS- H3.3 K27M and Ntv-a; p53 floxed mice with RCAS-H3.3 K27M + RCAS-Cre (20 mice per group).**

**Task 2 – Investigate potential cells-of-origin and developmental window for DIPG formation**
**Task 2a -** Infect the ventricular system of the posterior fossa of GFAP tv-a neonatal mice (20 mice) with RCAS-PDGF-B and observe for development of tumors

**This task has been partly completed. We have generated four tumors so far and a representative H&E of GFAP tv-a (Gtv-a) tumor is illustrated in Figure 5 (next to an H&E of a Ntv-a tumor which is quite similar histologically). Hydrocephalus mice have been**

**excluded from the analysis. Percent hydrocephalus for this experiment was 50%, which was due to leptomeningeal tumor.**

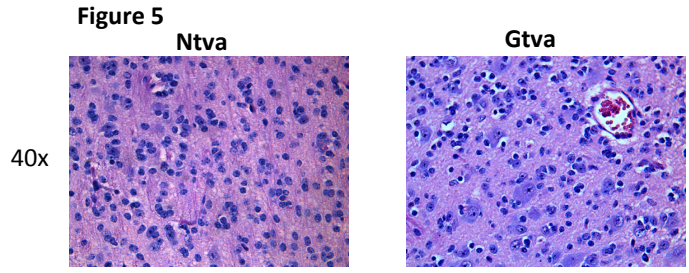



**Figure 5**- High magnification H&E of PDGF induced low-grade brainstem gliomas In Ntv-a and Gtv-a mice.

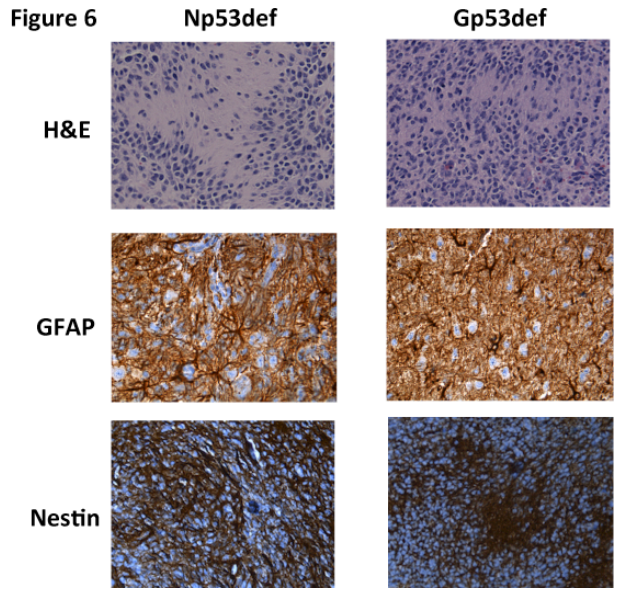
**Task 2b** - Infect the ventricular system of the posterior fossa of GFAP tv-a; Ink4a-ARF null neonatal mice (20 mice) with RCAS-PDGF-B and observe for development of tumors

**This task has not been completed. However, given the rarity of Ink4a-ARF deletions in DIPGs, we request to change this task. A new task is described below. Total mouse would be the same.**

**New Task 2b- infect the ventricular system of the posterior fossa of GFAP-tv-a; p53 $^{fl/fl}$ neonatal mice with RCAS H3.3 K27M together with RCAS-Cre and observe for the development of tumors (20 mice)**

**Task 2c** - Infect the ventricular system of the posterior fossa of GFAP-tv-a; p53$^{fl/fl}$ neonatal mice (20 mice) with RCAS-PDGF-B together with RCAS-Cre and observe for development of tumors

**This task has been completed and the survival curve for this mouse cohort was included in Figure 1. Out of 23 GFAP tv-a; p53 floxed mice infected with RCAS-PDGF-B and RCAS-Cre, 18 developed gliomas (78% success rate). Immunohistochemical characterization relative to Nestin tv-a model p53 floxed model is illustrated in Figure 6 (no significant differences in H&E or immunostaining for GFAP or nestin). Hydrocephalus mice were excluded from the analysis. The hydrocephalus rate for this experiment was 56% (due to leptomeningeal tumor).**



**Task 2d** - Infect the ventricular system of the posterior fossa of Ntv-a; p53 $^{fl/fl}$ neonatal mice with RCAS- PDGF-B and RCAS-Cre at postnatal D2, postnatal D7, and postnatal D14 (20 mice per group).

**This task is partly completed (postnatal D2 is complete). Postnatal D7 and postnatal D14 will be performed in year 2.**

**Task 2e**- Perform expression profiling of PDGF-B cortical tumors (5 tumors) and compare to results from task 1E in consultation with the DNA Microarray facility at Duke.

**This task has been completed. Our results identified Pax3 as a differentially expressed gene between brainstem gliomas (BSGs=DIPG) and cortical gliomas. We performed expression profiling of murine brainstem and murine cortical gliomas, induced by overexpressing PDGF-B in nestin expressing progenitors in the brainstem or cortex respectively of Ntv-a;Ink4a-ARF deficient mice. To our surprise, there were only 9 genes that were significantly differentially expressed between brainstem gliomas (BSG=DIPG) and cortical gliomas (CG) and significantly differentially regulated between normal brainstem (NBS) and BSG (table on the left). In this short list, we chose to focus on pax3; a transcription factor that is known to have oncogenic functions in neural crest derived tissues, and has already been implicated in having region-specific expression in ependymoma and pilocytic astrocytoma. Pax3 belongs to the paired box family of transcription factors. It is known to have regionalized expression in the developing neural tube and to confer positional identity to neural precursors (dorsal-ventral axis). Its expression and role in brainstem gliomagenesis are not known. Recently, Pax3 has been shown to inversely correlate with GFAP expression, a marker of differentiation in the astrocytic lineage. We confirmed that *pax3* mRNA is overexpressed in brainstem gliomas by real time RT PCR and also observed that Pax3 protein is overexpressed in brainstem gliomas relative to normal brainstem and cortical gliomas (Figure 7). We also confirmed that this region-specific expression of pax3 is in the tumor compartment by using Olig2-GFP mice. By sorting DIPG tumor cells in the Olig2-GFP background for GFP, we sorted for the tumor cell compartment and observed that *pax3* mRNA is mostly expressed in Olig-2 expressing tumor cells (data not shown).**

| Gene | Fold change BSG vs. NBS | P-value BSG vs. NBS | Fold Change BSG vs. CG | P-Value BSG vs. CG |
|---|---|---|---|---|
| Etl4 | -17.3 | 1.67E-08 | -3.20 | 1.65E-05 |
| Slc22a3 | 11.61 | 3.63E-08 | 3.56 | 5.89E-06 |
| Bmp4 | -2.57 | 3.47E-07 | -2.77 | 1.94E-07 |
| Chl1 | -7.21 | 2.66E-06 | -12.9 | 3.66E-07 |
| Pax3 | 4.96 | 1.04E-05 | 8.44 | 1.20E-06 |
| Irx5 | 3.34 | 0.00267 | 19.0 | 6.15E-06 |
| Pcyt1b | 2.49 | 1.11E-05 | 2.46 | 1.24E-05 |
| Myo5b | -2.41 | 0.00553 | -7.59 | 2.41E-05 |
| Ubtf | 1.97 | 2.30E-05 | 2.01 | 1.86E-05 |

Figure 7

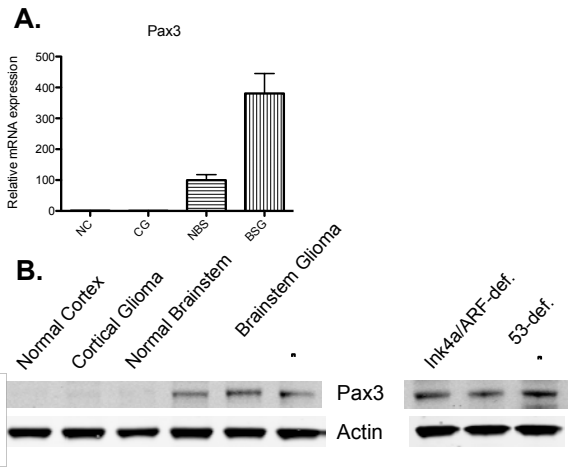



Figure 7. A. quantitative RT-PCR confirming that pax3 mRNA is overexpressed in brainstem gliomas (DIPGs). B. Western blot confirming that pax3 protein is also overexpressed in brainstem glioma (DIPGs)

**Key Research Accomplishments**

**1.** The establishment of improved DIPG animal models that can be used as preclinical tools.

**2.** We have observed that GFAP expressing cells in the brainstem can also be transformed to generate DIPGs.

**3.** We have generated cell-lines from the genetically engineered DIPG models that are being used as part of the DIPG preclinical consortium (together with several laboratories that are using human DIPG xenografts) to help prioritize the translation of novel agents for clinical trials for children with DIPG.

**4**. The identification of pax3 as a significantly differentially expressed gene between cortical gliomas and brainstem gliomas (DIPGs) that are driven by the same genetic alterations (PDGF and Ink4-ARF loss). This transcription factor is significantly upregulated in a third of human DIPGs (expression profiling data) and its role in gliomagenesis is currently being investigated in our group.

**REPORTABLE OUTCOMES**

**1.** DIPG mouse models that recapitulate the genetic alterations of the human disease

A. PDGF B; p53 deficient DIPG with nestin progenitors as cells-of-origin
B. PDGF B; PTEN deficient DIPG with nestin progenitors as cells-of-origin
C. PDGF B; p53 and PTEN deficient DIPG with nestin progenitors as cells-of-origin
D. PDGF B; p53 deficient DIPG with GFAP progenitors as cells-of-origin

**2**. Expression profiling of PDGF B; p53 deficient DIPG with nestin progenitors as cell-of-origin

**3**. Results from this grant served as preliminary data for a Damon Runyon Clinical Investigator Award application (based on results from task 2E) titled **Region-specific differences in CNS Gliomagenesis**, which was successful.

**4**. We have generated cell-lines derived from our models, which are being used as part of the DIPG preclinical consortium- a new consortium, which is helping the Children's Oncology Group prioritize the translation of novel agents into clinical trials for DIPG

**Conclusion**

Diffuse Intrinsic Pontine Glioma is an incurable tumor in 2012. This tumor is the leading cause of death in children with brain tumors. Our laboratory is one of few laboratories around the world that is studying this rare incurable tumor. Our approach is unique as we are using genetic engineered mouse modeling techniques to dissect the contribution of specific drivers to brainstem gliomagenesis, to identify the cell(s)-of-origin for DIPG formation, and to develop improved DIPG mouse models as preclinical tools. Our accomplishments so far are the development of improved DIPG mouse models, including a DIPG mouse model with a potential different cell-of-origin marker (GFAP as opposed to Nestin). In addition, we have identified pax3, a transcription factor that is significantly upregulated in our DIPG mouse model and not in a cortical glioma model that is driven by identical genetic alterations. As we start year 2 of the award, we will continue to improve our DIPG mouse models, move forward toward identifying a cell-of-origin for DIPG, and start preclinical testing with our DIPG mouse model. Several publications over the past few years have described the landscape of genetic alterations in this disease. As a result, we have requested to change two tasks to keep abreast with a significant discovery in the field -the identification of the K27M H3.3/H3.1 mutations in DIPG- since we initially submitted the application in 2010.

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